

Muscle disuse; the culprit of sarcopenia?

A study in master athletes

Michael Ewart Evans

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Manchester Metropolitan University

Faculty/Department – Science & Engineering

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Acknowledgement of Work Contribution

Due to the nature work carried out in the subsequent study, the contributions of different individuals are acknowledged in this section as not all lab work could be carried out individually.

Muscle biopsy sample harvesting along with preparation and fixation onto slides was carried out by Dr. Hans Degens and Dr. Marko Korhonen in Finland at the University of Jyväskylä.

Histochemistry and other experimental work were carried out by myself (Michael Evans) along with another masters student (Ali Akbar Fathnejad). Both contributed to the process of data acquisition in the form of digitizing traces.

Inter-rater reliability was calculated between both tracers for reliability purposes.

Data analysis was carried out by myself (Michael Evans) of the digitised traces and can be seen in the results section.

Information gathered on the master athlete cohort was done so by Dr. Marko Korhonen.

I would like to thank all people involved for their assistance in the following study.

1 – Abstract

Sarcopenia is described as the result of a slow but progressive age-related loss of skeletal muscle mass which is reflected by a reduction in the force and power generating capacity of the remaining muscle tissue. Ultimately the changes result in a transition from an independent to dependant lifestyle and presents a growing issue particularly in more economically advanced countries where an ageing population and changing demographic is apparent. An age-related decline in physical activity levels means that muscle disuse is more prevalent with advancing age and therefore the sarcopenic phenotype is more apparent. By studying a unique population who maintain very high physical activity levels exhibiting exceptional athletic ability even in very old age, the aging process can be studied without the confounding factor of disuse seen in sedentary individuals. Through histochemical cross-sectional analysis of muscle biopsies collected from these individuals ten years apart, differences can be seen in how muscle tissue changes with advancing age compared to how muscle tissue of sedentary individuals changes. Muscle fibre morphology and capillary supply of the muscle tissue was found to be better maintained than is normally seen in sedentary individuals of comparable age with no significant changes seen over the ten year period where normally declines would be expected to be observed.

2 - Aims & Objectives

The aim of the study is to understand the contribution age-related decline in physical activity levels has upon the ageing skeletal muscle phenotype and to the process of sarcopenia.

3 - Introduction

3.1 – Skeletal Muscle Physiology

3.1.1 – Overview

Muscle tissue is responsible for generating all movement, both voluntary and involuntary, in animals and is therefore absolutely essential to life. Skeletal muscle is highly striated and is responsible for allowing contraction and subsequent relaxation to occur. It is the process of contraction in muscles that converts chemical energy into movement energy and this transfer is recognised by the movement of the bones within the body. Motor unit activation in healthy skeletal muscle can be finely regulated to allow precise control over the force generated by the muscle. Tendons allow this transfer to occur

as they are constituted of highly compact bundles of collagen connecting the skeletal muscles to bones allowing the process of movement to be carried out effectively along with other connective tissue playing key roles in maintaining the shape and structure of the muscle to facilitate contractility. There are two key tubular networks within muscle tissue that play a key role in signal transduction required for contraction. The first of these being T-tubules that allow a signal (action potential) for movement to penetrate deep into and

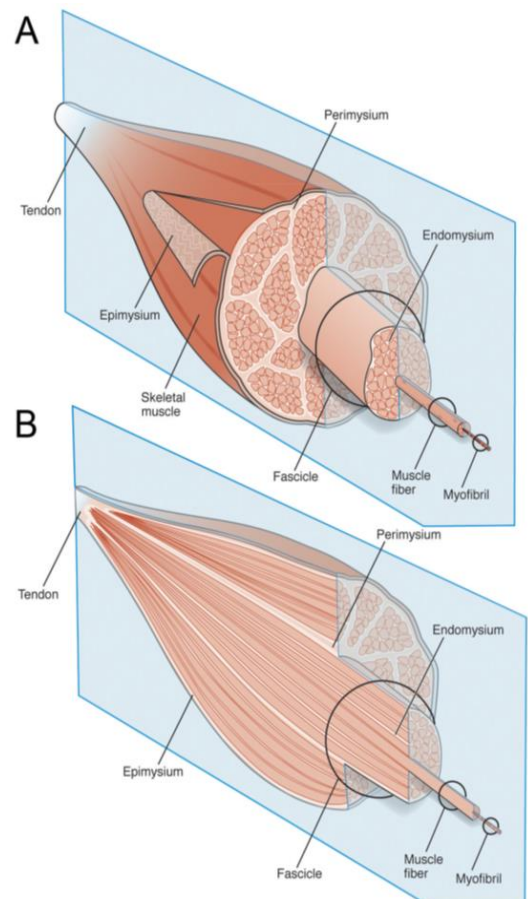


Figure 3.1: *Diagram representing hierarchical organisation of skeletal muscle including the ECM structures. Figure taken from (Gillies and Lieber, 2011.)*

throughout the muscle fibres to initiate contraction and the second being the sarcoplasmic reticulum (SR) which upon stimulation allows for the efflux of Calcium ions (Ca^{2+}) triggering changes in the sarcomeric structure permitting contraction and the reverse process allowing subsequent relaxation.

Skeletal muscle itself is a highly hierarchical tissue and this structure allows for a wide range of movement. The smallest unit of the muscle is individual myofibrils which are compacted very tightly in slightly larger units known as muscle fibres. These are further grouped into fascicles, of which again, many exist within a single muscle. The muscle fibres are closely associated with relatively little extra-cellular matrix (ECM) present between them allowing for a dense areas of functional tissue which constitutes to the bulk of the skeletal muscle tissue. A larger proportion of the ECM is found encompassing fascicles and the larger units of the muscle and the ECM within skeletal muscle is mainly found as connective tissue providing scaffolding and support for the growth, development, repair and function of the tissue.

3.1.2 – Myofibrils and Key Proteins

The striated appearance of muscle previously mentioned is highly apparent when looking at skeletal muscle at the myofibre level. Several key proteins are present within myofibres, and through their precise arrangement a contraction can be initiated to occur simultaneously throughout the whole muscle to convert what would be a minute movement when considering the individual protein-protein interaction, into a significant force allowing movement.

Work on determining the make-up of the individual repeated contractile unit, the sarcomere, within skeletal muscle was carried out by using high angle x-ray

diffraction at the University of Leeds (Astbury, 1947). Two distinct bands were identified based on their different refractive indices; light bands (I-bands) with a

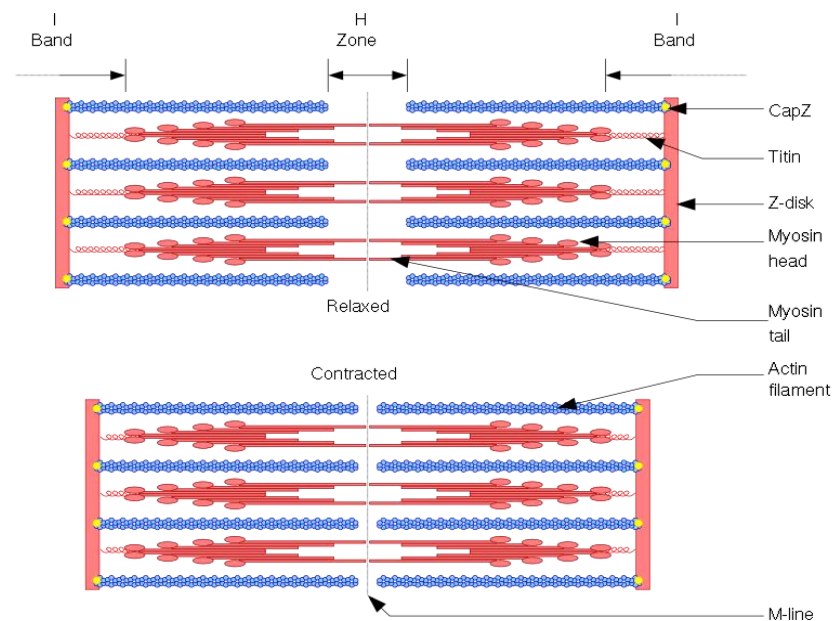


Figure 3.2: Diagram of the repeat contractile unit of skeletal muscle – the sarcomere - with key proteins and the interlocking arrangement that gives rise to the striated appearance of muscle is displayed.

low refractive index which make up the thin filaments were determined to be comprised of the protein actin while dark bands (A-bands) with a high refractive index are the thick filaments containing the protein myosin (Figure 3.2). Several other proteins are also essential to the sarcomeric structure most notably titin, troponin and tropomyosin.

Actin and myosin filaments are arranged in a parallel manner allowing for many contacts to be made by the numerous myosin heads that exist within a myosin filament with the binding sites present on actin filaments. Actin filaments are bound to z-discs at either end of the sarcomere and protrude inwards toward the centre of the sarcomere known as the M-line. Myosin molecules aggregate under normal physiological conditions allowing for the myosin head (responsible for the interaction with actin) to be presented on the exterior of

the myosin filaments while the myosin tails pack tightly together (Huxley, 1974). Titin is a large elastic protein that interacts with both actin and myosin as well as both z and m lines. It plays a key role in maintaining sarcomeric structure as well as generating passive tension – functions the ECM also assists (Peckham et al., 1997) (Anderson and Granzier, 2012). It is also essential for stabilising the myosin filaments and aligning them with the centre of the sarcomere. Troponin and tropomyosin are two proteins that interact to form a complex that controls the process of the myosin heads binding to actin by blocking the binding sites present on the actin filaments. When stimulation occurs in the form of an action potential the sarcoplasmic reticulum release Ca^{2+} ions which cause structural changes in the troponin-tropomyosin complex exposing myosin binding sites on the actin filaments.

Following completion of the previously described processes the myosin head then is able to undergo hydrolysis of adenosine triphosphate (ATP) which allows the characteristic power stroke to occur in which the myosin head binds to binding sites on the actin filaments and pulls itself along the neighbouring actin filament before releasing and the process can repeat again now binding further along the actin filament. The end result of this process is the z-lines being pulled closer together and the sarcomere is shortened hence causing a muscular contraction. This model of movement is known as the sliding filament model and was elucidated by work carried out independently in 1954 by Hugh Huxley and Jean Hanson and also Andrew F Huxley and Rolf Niedergerke (H. Huxley and Hanson, 1954; A. F. Huxley and Niedergerke, 1954) .

3.1.3 – Motoneurons and Motor Units and Fibre Typing

Motor neurons (or motoneurons) are nerve cells that are responsible for transmitting the nerve impulse or action potential responsible for causing a resulting contraction as described previously. Motoneurons run from the central nervous system to muscles with up to several hundred innervating an individual muscle. Motoneurons have a much specialised design similar to that of other neurons with the soma (cell body) which contains the nucleus of the cell, dendrites attached to the soma with branched projections extending outwards in order to propagate electrochemical signals received from other neural cells toward to soma, and also a long axon surrounded by a myelin sheath to allow the signal to be transmitted to the effector – in this case the skeletal muscle. Specialised synapses exist between muscle fibres and the axonal stem and these neuromuscular junctions rely on the neurotransmitter acetylcholine to chemically link the two separate entities conveying the appropriate message.

A singular motoneuron can effectively innervate many muscle fibres and therefore the cluster of muscle fibres being supplied by a singular motoneuron can be deemed to be acting in cooperation. This functional unit was termed a motor unit (Sherrington, 1929) and these motor units have been seen to vary greatly in the size and both the physiological and biochemical properties they exhibit.

When studying different fibre types physiologically intracellular microelectrodes can be used to excite individual motor units and their twitch contraction times and fatigability measured (Burke et al., 1973) which led to three main different

fibre types being identified. Fast fatigable (FF) fibres capable of high force rapid contractions that fatigue very quickly; Fast fatigue resistant (FR) fibres that have a fast contraction speed, intermediate force but are more resistant to fatigue; and slow oxidative (SO) fibres that carry out low force, slower contractions but are highly resistant to fatigue (Intermediate types are also found to exist between FF and FR) (Pette, 1985).

These findings are further supported by histochemical studies that are devised to study the different enzymatic activity of these fibre types. The SO fibres described previously were found to be high in oxidative enzymes and low in glycolytic enzymes as well as having a slower myosin ATPase activity. The FF and FR fibres are more similar to one another as expected containing high glycolytic enzyme levels and myosin ATPase activity with the main difference being that the FR fibres contain higher oxidative enzyme levels than FF fibres. These differences allow them to be visualised histochemically using staining techniques.

In addition to this more recent immunohistochemical techniques focus on the presence of different myosin heavy chain (MyHC) isoforms within the myosin molecules (Reiser et al., 1985). It is the heavy chains in myosin that determine the cross-bridge kinetics and therefore the speed of contraction (Bottinelli et al., 1991) and because of this different antibodies can be applied while staining to visualise different MyHC isoforms present and therefore identify different fibre types. SO fibres with low myosin ATPase activity levels contain the MyHC type I isoform while the FR and FF fibres contain the different MyHC isoforms type IIa and IIx respectively.

Different types of motor units are recruited dependant on the type of contraction and task required of the given muscle and can be activated in order to further fine tune the output of a given muscle.

It is important to note that processes of denervation and re-innervation can occur as motoneurons contact with muscle fibres changes. A muscle fibre will normally undergo atrophy following the loss of innervation from a muscle fibre however neighbouring motoneurons can prevent the muscle fibre atrophy if they manage to re-innervate the denervated muscle fibre prior to this process occurring.

3.1.4 – Connective Tissue, ECM and Capillarisation

The muscle and the neurons that supply it alone are not enough to effectively carry out muscle contractions. The connective tissue and ECM surrounding the muscle tissue are essential to provide not only scaffold for correct development and maintenance of the overall structure. It is also responsible for supplying a source of satellite cells required for the original development and any repair that is required as the muscle undergoes hypertrophy or damage. Satellite cells retain a regenerative capacity and remain in a quiescent state while located in the basal lamina layer of the ECM surrounding individual myofibrils (MAURO, 1961). Upon correct stimulus from myogenic regulatory factors (MRFs) these cells re-enter the normal cell cycle allowing regeneration of new myoblasts to repair damaged or form new myofibres (Sinanan et al., 2006) (Jejurikar et al., 2006). MRFs play a key role in all signalling processes occurring within the muscle, not just with satellite cells, allowing for the production of the essential proteins and other components that make up muscle tissue.

The transmission of the contractile force is also reliant on connective tissue ensuring that the muscle tissue is effectively supported to allow the directional simultaneous contraction of the whole muscle causing movement of the desired skeletal component through tendons.

Another key function of the loose connective tissue is to provide a space for blood vessels and nerves to supply the muscle fibres. The perimysium, which exists between muscle fibres, allows capillaries to supply muscle fibres and be in very close contact with contracting muscle fibres. The energy that is required to drive muscular contractions is derived from ATP hydrolysis and therefore ATP is required. Cellular respiration is the process by which ATP synthesis occurs and is a highly efficient process when an abundance of oxygen is present as oxygen is the terminal electron acceptor in the electron transport chain, a process carried out within mitochondria. Therefore a good supply of oxygen is essential for sustained periods of muscular work and oxidative phosphorylation to form ATP. Alternatively ATP can be produced by anaerobic glycolysis when oxygen supply is limited however this cannot occur for long periods of time due to the build-up of lactic acid in muscles, the by-product of this process. The demand for a highly effective supply is obviously much greater in muscles than in many other tissues due to the nature of the function of the tissue and the process required to realise that function.

Muscle tissue is highly plastic and adaptable and will undergo changes based upon the stresses and demands put upon it. One of the key and adaptable features is that of the oxygen supply as an efficiently functioning muscle will require an extensive and well distributed network of capillaries and blood

vessels to ensure that muscle contractions can occur via the process of ATP hydrolysis.

3.2 – Sarcopenia

3.2.1 - Overview

As has been discussed previously skeletal muscle is a highly adaptive and precisely arranged tissue, and for correct and proper functionality, it is heavily reliant on all of the different aspects of the tissue being operational and in good working order. This system however begins to deteriorate with age and leads to the condition known as sarcopenia presenting itself within skeletal muscle.

Sarcopenia is described as the result of a slow but progressive age-related loss of skeletal muscle mass and function. In addition to the age-related muscle atrophy, or overall decrease in muscle size, there is a reduction in the “quality” of muscle tissue. This is reflected in the muscle by an overall reduction in the force and power generating capacity of the remaining muscle tissue. Alterations to the normal cross-bridge kinetics required for the efficient contraction as portrayed by the sliding filament model (Huxley, 1974) as well as increased levels of fibrosis and a transition from fast to slow fibre type composition (Larsson and Ansved, 1995) are in part responsible for the deterioration of the quality of muscle tissue. Other factors such as inflammatory processes, oxidative stress, neuromuscular degeneration as well as satellite cell dysfunction inhibiting the regenerative capacity of skeletal muscle also contribute to the change in muscle phenotype and function (Degens et al., 1998; Degens and Alway, 2003; Degens, 2010).

Ultimately, the changes will result in frailty and a transition from an independent to dependant lifestyle where more help and assistance will be required to carry out basic tasks. Furthermore the risk of accidents and injury occurring from tasks or simple movement that a normal healthy individual would not struggle with is significantly increased. This is a growing issue particularly in more economically developed countries as average life expectancy is on the rise and the increasing ageing population where the sarcopenic phenotype will be more prevalent .

3.2.2 – Fibre Type Transition

The muscle wasting during ageing is attributable to both a loss of fibres and fibre atrophy that starts at around the age of 50 (Lexell et al., 1988). The atrophy appears to affect type II fibres more than type I fibres (Larsson et al., 1979; Klitgaard et al., 1990). As a result, the areal proportion of type I fibres in the muscle increases. The myosin ATPase activity is positively related to the maximal shortening velocity of a muscle (Barany, 1967) and hence the age-related fast-to-slow transition in areal fibre type composition may contribute to the slowing of the muscle in old age. The fibre cross-sectional area (FCSA) is main determinant of maximal force output and so atrophy of muscle fibres would explain to a large extent the age-related loss of muscle strength. This is, however, not the whole story, as also the specific tension (force per muscle cross-sectional area) is reduced in old age at both the whole muscle and single fibre level (Degens et al., 2009). The loss of force and slowing of the muscle will have a significant negative impact on the power generating capacity of the muscle in old age, as power is force times velocity. The reduction in specific

tension may be related to a reduced myosin concentration in the muscle (d'Antona et al., 2003) (Faulkner et al., 2007), while the slowing of the muscle may be related not only to changes in fibre type composition, but also post-translational modification of myosin (Ramamurthy et al., 2001).

It is generally accepted that there is a greater impact upon type II fast twitch muscle fibres during the normal ageing process (Lexell et al., 1988). FCSA Studies have shown that type I muscle fibre size appears to be largely unaffected during ageing and that the main contributor to the decrease in muscle mass is the atrophy of smaller type II fibres (Larsson and Karlsson, 1978; Orlander et al., 1978; Verdijk et al., 2009; Klitgaard et al., 1990). The resultant effect of this is a decline in the ratio of areas of type II to type I fibres (Lexell et al., 1988). Some studies, particularly those focusing on the determination of MyHC isoforms from gel electrophoresis, suggest there is a shift toward the expression of the type I MyHC isoform but this may be due to the nature of the selective atrophy of the type II muscle fibres making the overall percentage of MyHC type I containing fibres appear increased (Short et al., 2005).

The atrophy observed of muscle fibres is associated with the progressive loss of motor neurons which causes muscle fibres to become denervated. While re-innervation occurs to some degree this process is not substantial enough to prevent the loss of muscle fibres and therefore atrophy occurs. It is this loss of normal neural function that appears to be more significant in type II fibre supplying neurons and the re-innervation process appears to cause fibre type grouping mainly of type I fibres and helps explain the transition (Ansved, 1991).

While the general opinion is that muscle fibre atrophy occurs and does so to a greater extent in type II compared to type I fibres, the specific ages at which this occurs and to what extent has been shown to vary in different studies ranging from it only occurring at very old age (Aniansson et al., 1992) to no significant changes being observed. Other studies however contradict these findings and in some cases such as a longitudinal 12 year study an overall increase in percentage type II fibres was observed (Frontera et al., 2000). Observed increases in FCSA are probably due to compensatory hypertrophy occurring as muscle fibres atrophy and neighbouring fibres undergo hypertrophy to retain the muscle size and function. This may be due to differences in exercise, lifestyle and general health and is a reason that more studies into these changes is required.

3.2.3 - Changes in capillarisation

Microvascular adaptations and in particular capillary supply is a highly dynamic process with significant alterations in factors that affect the degree of tissue oxygenation (such as capillary density (CD) and local capillary to fibre ratio (LCFR)) seen during ageing, disuse and exercise training (Proctor et al., 1995). The capillary supply is therefore highly important to have an understanding of when considering efficient muscle function. Different methods have been applied when trying to determine the quality of capillary supply and therefore tissue oxygenation. By simply considering the arrangement of capillaries around muscle fibres, as was conducted in early studies, it was found that the more oxidative fibres (type I and type IIa) had a greater capillary to fibre ratio (C:F) than more glycolytic fibres (type IIb). Limitations of this method include

the fact that, while direct contact of capillaries around a fibre will oxygenate the fibre, direct contact is not essential as diffusion can occur further than this method allowed for. Furthermore these methods also do not account for variations observed in fibre sizes. A better method is that of capillary domain (described in methods section) where the heterogeneity of capillary spacing (Log_{RSD}) and both direct and indirect contacts of capillaries to fibres or varying sizes can be considered (Degens et al., 2002; Wüst et al., 2009). When taking into account the local metabolic environment in this way as well as the fibre size a significant correlation between capillary density and overall fibre size has been shown to be more apparent and also to be independent of fibre type (Degens and Veerkamp, 1994). The degree of heterogeneity of capillary spacing has been shown to increase with ageing in rats and as the variation in fibre size too increases indicating reduced whole tissue oxygenation levels (H. Degens et al., 2009). An increase in heterogeneity of capillary distribution would be expected to negatively impact tissue oxygenation (Degens and Korhonen, 2012). The reason for this is that a homogenous distribution of capillaries and fibre sizes will allow for oxygen to be distributed to all areas of the muscle tissue effectively and thus, allow for effective contraction of the muscle. If there are areas lacking proximity to a capillary the oxygen supply can be reduced. Also a large variety in fibre sizes can mean that oxygen diffusion distances will vary to a greater extent and therefore tissue oxygenation levels may not be consistent across the entire tissue.

FCSA is positively correlated with local capillary to fibre ratio (LCFR) in humans and rats (Degens et al., 1994; Wüst et al., 2009) rather than the oxidative

capacity of the fibres themselves suggesting that the compensatory hypertrophy occurring as the denervation-re-innervation process occurs has a greater impact upon capillarisation. During ageing changes occur to this environment hence causing the reduction of force generating capacity and decline in muscle function.

3.2.4 – More Observed Changes

As mentioned previously, the muscle tissue environment is highly plastic and relies on constant regenerative processes ensuring the maintenance of the muscle tissue. The presence of reactive oxygen species, which are increased in older age as systemic inflammatory processes occur, can cause mitochondrial dysfunction along with alterations to gene expression and hormonal levels. An alteration to gene expression has an impact upon the efficacy of MRFs and their ability to initiate transcription of genes under their control. This will result in alterations to the functionality of satellite cells will be apparent which will undoubtedly have an effect of muscle growth and regeneration (Chargé et al., 2002). This therefore means that chronic low-grade systemic inflammation also plays a key role in the age-related decline of muscle mass and function (Degens, 2010).

3.3 – Master Athletes

While skeletal muscle atrophy cannot be completely reversed in old age because of the loss of fibres (Degens, 2012), the extent of fibre atrophy is dependent on the physical activity level of the individual (Faulkner et al., 2007) and muscle mass can be increased with resistance exercise even in the old (Frontera et al., 1985). Also improve the aerobic capacity of the muscle can be

improved with endurance training (Pollock et al., 1997). It is therefore expected that muscle size and function will be better maintained in master athletes than in age-matched controls.

An important and modifiable factor that is well known to cause muscle wasting is disuse (Degens and Alway, 2006). Given that ageing is associated with a decline in physical activity levels, it is of interest to know to what extent the decline in physical activity levels contributes to the age-related changes in muscle.

In order to gain better understanding of the contribution of disuse to sarcopenia, a unique population of individuals; master athletes are of particular interest. Master athletes maintain very high physical activity levels, with exceptional athletic competition and achievements even in very old age (Faulkner et al., 2008). This suggests that their high levels of exercise does indeed help to preserve muscle mass and acts as an effective counter measure against the normal age-related muscle wasting and dysfunction, as described previously, seen in sedentary individuals (Wroblewski et al., 2011). Master athletes are therefore an ideal population to study the ageing process per se, without the confounding factor of disuse seen in sedentary individuals.

A better understanding of the processes underlying sarcopenia is of the utmost importance for the design of programmes or life style changes that will allow people to retain an independent lifestyle into old age. If prevention, attenuation or reversion of age-related muscle wasting and dysfunction is possible then individuals will be able to retain a higher degree of mobility and independence into later life (Butler-Browne et al., 2013).

Exercise is known to have beneficial effects on the levels of chronic low-grade systemic inflammation (Degens, 2010), help attenuate the loss of motor units (Degens and Korhonen, 2012), maintain overall fibre function and preserve the satellite cell pool and functionality (Kadi and Ponsot, 2010). These effects can be seen even in old aged individuals with the skeletal muscle responding to resistance training displaying an increase in muscle mass and strength (Morse et al., 2005). All of these features indicate that regular exercise will help to preserve muscle function and the overall state of the muscle leading to successful muscle ageing rather than the normal declines observed in sedentary individuals.

4 – Methods and Materials

4.1. Subjects

4.1.1. Master Athletes

A study into the effects of age and long-term sprint training on muscle structure and function (Korhonen et al., 2006) utilised a group of male sprint-trained athletes (ages 40-89) based in Finland. To qualify for classification as a master athlete, individuals were required to have a long-term sprint training background and have competed at athletic championships, nationally or internationally, in sprint distance events (100m – 400m sprinting events).

Subjects involved in this study were recruited and written consent obtained in line with a previous study (Korhonen et al., 2006) which gained approval from the Ethics Committee of the University of Jyväskylä and conformed to the Declaration of Helsinki. All master athlete subjects who contributed to this study were members of Finnish track and field organizations and were presumed healthy partly due to their lifestyle choices and confirmed by reference to their detailed medical history and interviews conducted. Height and weight information was also recorded for all participants.

Twenty-two individuals provided muscle biopsies that were analysed for a longitudinal 10 year study. The initial biopsy was taken in autumn 2002 with another biopsy taken in autumn 2012. This means that there were biopsies and information available at two different time points separated by ten years. For ease of reference these will be referred to hereafter as shown in Figure 4.1.

Biopsy	Date taken	Referred to as:
Initial biopsy	Autumn 2002	Pre-Study Samples
10 year follow up biopsy	Autumn 2012	Post-Study Samples

Figure 4.1 - Table displaying different biopsies used for this project and how they will be referred to

At the time of the first biopsy 5 of the subjects were aged between 40-50 years old, 6 subjects fit into each age band 50-60 years old and 60-70 years old and a final five were aged between 70 and 80 years old. The subjects were grouped in this way to allow for analysis of data between the different age groups. Issues can arise when interpreting data in this was as the continuous variable of age is broken into discrete categories which can result in two subjects being placed into different groups even though a very small difference in age is present.

4.1.2. Muscle Biopsy

Biopsies were taken from the vastus lateralis, the largest part of the quadriceps femoris, of the individual's dominant leg. Leg dominance was determined by the subject's knowledge of their own athletic ability and strength testing. This muscle was chosen as it is a key determinant in sprint performance. The experimental procedure has been previously described (Korhonen et al., 2006). In brief, a needle microbiopsy technique (Hayot et al., 2005), adapted from a widely used but more invasive needle biopsy technique (Bergström, 1962), was applied with suction ensuring insertion to the middle region of the muscle at a depth of 1.5-2.5cm. Attempts to keep the depth of biopsy as consistent as possible were important to eradicate the potential for variation which can be seen between biopsies taken from superficial, mid and deep region of the muscle (Dwyer et al., 1999). This ensured that data collected from the biopsy

will be representative of the individuals muscle characteristics rather than a product of an inconsistent biopsy technique. Muscle samples were then cleaned of visible unwanted connective and adipose tissue before being divided up and treated dependant on the intended future use.

Part of each biopsy was immediately frozen in liquid nitrogen and stored at -80°C to be used for biochemical analysis of myosin heavy chain isoforms. A further subsection was examined under microscope to determine the dominant fibre orientation before being mounted to ensure sections cut from the biopsy would be cross-sectional. An embedding medium was used to help stabilise the biopsy on a cork disc followed by subsequent freezing in isopentane to -160°C by using liquid nitrogen. Samples were then stored at -80°C until use.

4.2. Muscle Biopsy Methodology

4.2.1. Histochemistry

The portion of the biopsy that was mounted to ensure fibres were orientated transversely, as described previously, was cut to produce many 10µm thick serial sections using a cryostat (*Leica CM 3000*) cooled to -24°C which were then mounted onto glass slides (Korhonen et al., 2006). Attempts to mount three sections per glass slide were made to improve likelihood of a successful staining procedure. These slides were frozen at -80°C until use.

Due to the nature of the subsequent work (4.3.) that was required to be carried out, a co-staining protocol was applied. This was designed to allow visualisation of capillaries and fibres simultaneously with distinction between type I and type II fibres also visible. This made generating a digitised trace of the cross-section

from a single stained section (4.3.1). This protocol was adapted from previous work (Degens et al., 2002).

Slides to be stained were removed from the -80°C freezer and dried under a stream of clean air. A PAP-pen was used to draw a hydrophobic circle around the samples to reduce quantities of staining fluid required. Sections were initially fixed in ice cold acetone for 15 minutes. These were then incubated in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH7.5) before being blocked for 45 minutes with 0.1% Bovine Serum Albumin (BSA). This was washed off using more HEPES buffer before samples were transferred to an opaque iron box so the staining could be carried out without interference of light. A peroxide incubation (1% (vol/vol) of 30% H₂O₂, 1x PBS and 2% (vol/vol) of 10% Triton-X-100) was applied to all slides (approximately 200µl per glass slide) for 30 minutes.

Following incubation solutions were washed using HEPES buffer from the slide before the co-stain incubation was applied. The co-stain contained the following components:

- Lectin (Biotinylated Ulex Europaeus Agglutinin I (*Vector Labs*) at 50 µg/ml (1:40)
- Anti-MyHC Type I Antibody (A4.951 myosin (*DSHB*)) at 1:200
- HEPES 1% BSA solution

This was applied to the slides and left for 60 minutes. Several washes with HEPES buffer followed before an anti-mouse secondary antibody (Vectastain secondary anti-mouse IgG antibody) (*Vector Labs*) was made up in HEPES 1% BSA solution (1:200) and left on the sections for another hour. Again several

washes in HEPES buffer were carried out following completion. Prepared 30 minutes in advance as instructed to, an ABC-solution from the Vectastain Elite ABC kit (*Vector Labs*) was added to each of the sections and left for 40 minutes. Subsequently the solution was thoroughly washed off using HEPES buffer several times before using the VIP substrate kit (*Vector Labs*) which was applied to all sections and the process followed under a microscope to ensure staining of the correct intensity. Once desired staining intensity was achieved the process was halted by washing in pure water for 5 minutes. Finally sections were embedded in a gently warmed glycerine-gelatine solution and glass coverslips fixed on top to prevent any further reactivity.

4.2.2. Myosin Heavy Chain Composition Gels

A portion of each muscle biopsy was treated as described above to allow for determination of the relative myosin heavy chain (MyHC) isoforms present. Muscle biopsy samples were kept on ice as they underwent homogenisation using a tissue grinder. The resulting homogenate was contained in a buffer solution (*sucrose 210mM, HEPES 30mM, EGTA 2mM, NaCl 40mM, EDTA 5mM pH 7.4 with supplementation of a 1% protease inhibitor (Sigma)*) before being centrifuged and the pellet resuspended in a buffer to solubilise membrane-bound components (*KCl 175mM, EDTA 5mM, Tris 20mM, Triton X-100 0.5%, pH 6.8*). Successive rounds of centrifugation and resuspension of pellets were carried out in another buffer (*KCl 150mM, EDTA 5mM, Tris 20mM pH 7.0*) which would allow for quantification of protein concentration of each homogenised sample (Hayot et al., 2005). Following this between 10 and 15 of the 10µm serial cryosections from each biopsy (processed described previously (see

4.2.1)) were placed in a lysine buffer containing 10% glycerol, 5% 2-mercaptoethanol (β -MEtOH), 2.3% SDS in a 62.5mM TrisHCl buffer (*pH* 6.8) (*total volume 700 μ l*) (Korhonen et al., 2006). This was then heated to 60°C for 10 minutes before being frozen at -80°C. Before small aliquots were applied to individual gel lanes (10-15 μ l) samples were boiled once for 2 minutes at 100°C. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) techniques were applied to resolve the different myosin isoforms present (I, IIa and IIx) and their relative quantities. These were adapted from previously used protocols (Korhonen et al., 2006; Andersen and Aagaard, 2000). A separating gel containing 6% acrylamide (*Sigma*) and 30% (vol/vol) glycerol (*Fischer*) as well as a TrisHCl buffer solution (1.5M *pH* 8.8) and SDS was polymerised using ammonium persulphate (APS) (*Sigma*) and tetramethylethylenediamine (TEMED) (*Sigma*). This was loaded into a maxi-gel dock and a stacking gel containing 4% acrylamide, glycerol and a TrisHCl buffer (0.5M *pH* 6.8) was cast on top of the separating gel. TrisHCl Buffers were thoroughly checked as slight change in the *pH* can affect electrophoretic properties of the gel. 10-15 μ l of each sample were loaded into individual gel lanes once fully set and the gel was lowered into the maxi-gel chamber containing a Tris-Glycine-SDS (TGS) (*Bio-Rad*) running buffer. Once in place the upper chamber was secured in place and filled with an upper buffer again made up of TGS but containing β -MEtOH. 0.07% β -MEtOH was originally used but this was increased to 0.12% to try and improve resolution of the bands (Kohn and Myburgh, 2006). An alternate cross-linking agent to the normal acrylamide-bisacrylamide combination was used to attempt

to reduce background staining. Piperazine di-acrylamide (PDA) (*Bio-Rad*) was used as this cross-linking agent (Rabilloud et al., 1997).

The gels were subsequently run on an electrophoresis device (*Bio-Rad*) at a constant voltage (120v) for 24 hrs. Attempts were made to improve isoform resolution by increasing the voltage as high as 275v. The gel system was constantly cooled using a water cooling device to 10°C.

Following completion of the run the gel was removed and transferred to a fixative solution (*Bio-Rad*) where it was left overnight. Fixative was thoroughly washed out of the gels by using several high purity water washes. A silver stain kit (*Bio-Rad*) was then used to visualise bands and once the staining was complete (10-30 minutes) the gel was transferred to a 5% acetic acid stopping solution. After 15 minutes the gel was again thoroughly washed before being dried using compression gel drying sheets (GE Healthcare Bio-Science) where it could subsequently be imaged using a Bio-Rad imaging device to check relative proportions of the three MyHC isoforms.

Due to the high molecular weight (~220 kDa) a lower percentage gel is ideal for resolving high molecular weight proteins so acrylamide percentages were trialled ranging from 5% to 7% however 6% provided the best balance between resolution and integrity of the gel itself. Unlike in rats (Pereira Sant'Ana et al., 1997) the MyHC isoform type IIx migrates the slowest and this is followed by type IIa and finally type I which migrates the furthest down the gel.

4.3. Cross-Sectional Morphology and Capillarisation Studies

In order to be able to collect data of fibre cross-sectional areas (FCSA) as well as the level capillarisation - defined by parameters such as capillary density (CD),

capillary to fibre ratio (C:F), heterogeneity of capillary spacing (Log_{RSD}) and how many capillaries supply each fibre (Domains around a fibre (DAF) and Local capillary to fibre ratio (LCFR)) – sections on muscle biopsies that have been stained need to be first imaged and then, a digitised trace produced allowing calculation of these parameters using a software program called Anatis.

4.3.1. Digitisation

Images of stained sections were taken at 10x magnification using a microscope and imaging software. Using a digitising tablet (model MMII 1201, Summagraphics, TX) fibre outlines of different types and relative capillary co-ordinates were traced from photomicrographs. The process is simplified in figure 4.2.

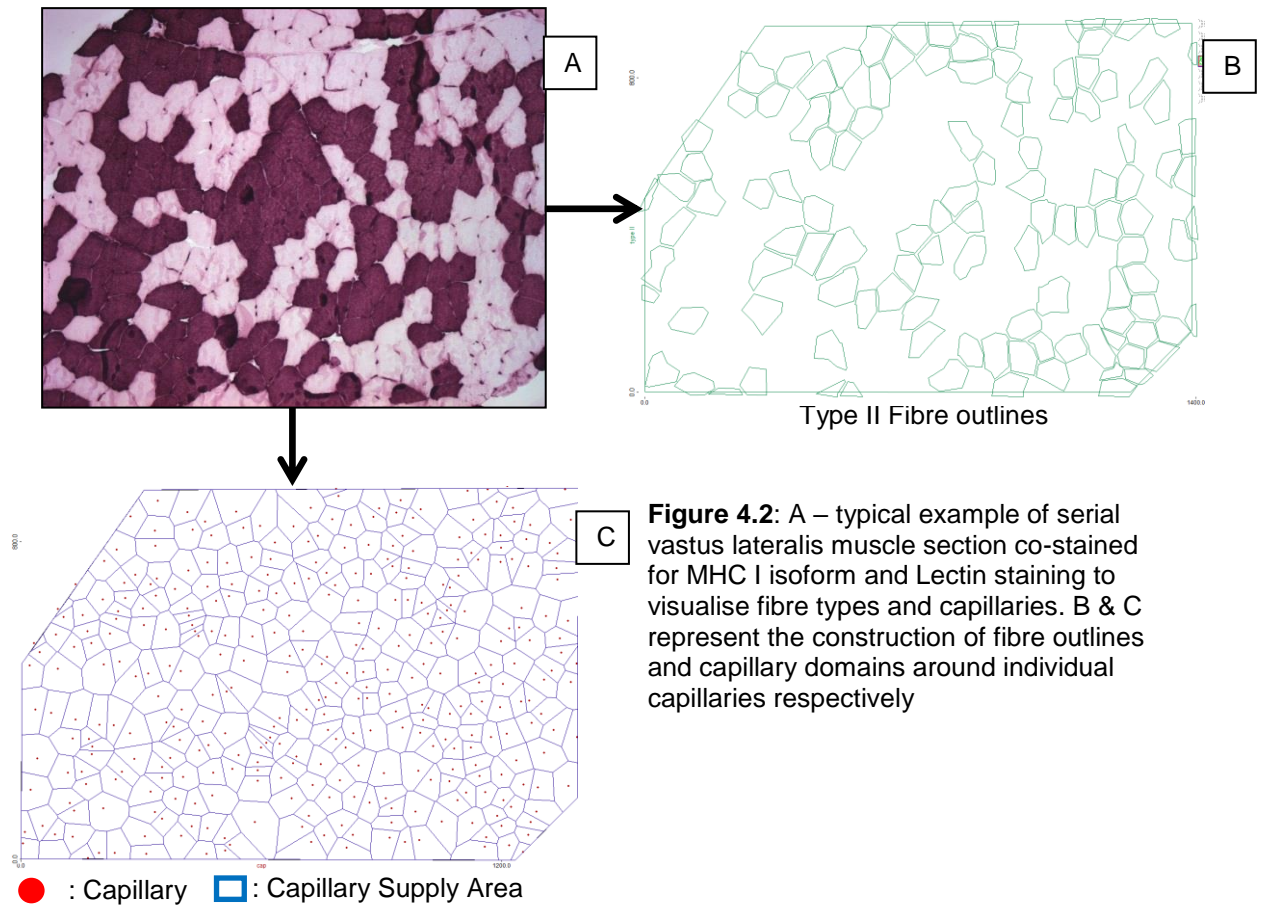


Figure 4.2: A – typical example of serial vastus lateralis muscle section co-stained for MHC I isoform and Lectin staining to visualise fibre types and capillaries. B & C represent the construction of fibre outlines and capillary domains around individual capillaries respectively

4.3.2. Capillary Domain Analysis

A method of capillary domains was used to analyse capillarisation within stained sections of muscle tissue (Degens et al., 1992; Hoofd et al., 1985; Degens et al., 2002). This method allows for much more insightful parameters to be calculated rather than just CD and C:F as it takes into account the capillary supply with respect to different fibre types and heterogeneity of capillary distribution throughout the muscle section (Degens et al., 2002). This is important to do as oxygenation of the muscle is of interest in this study and this method allows accurate determination of these parameters which have a large impact on this process (Degens et al., 1994).

FCSA and perimeters for fibres was determined using only complete fibres outlines as incomplete fibres on the border of the trace will not provide

accurate information. MyHC type composition of the fibres was calculated by working out both the percentages of different fibre types present as well as areas that the different fibre types take up. The percentage of the trace taken up by connective tissue was calculated by subtracting the total fibre area for each image from the total picture area. Each capillary is individually located and a “domain” is constructed around each of these which is defined as the area surrounding a capillary delineated by equidistant boundaries from adjacent capillaries (Degens et al., 2002; Hoofd et al., 1985). These are known as Voronoi polygons and eradicate the normal limitations of methods where only capillaries in direct contact with fibres are deemed to supply said fibre. For the same reason as when calculating FCSA and perimeters of contours, domains in contact with the edge of the image (i.e. incomplete domains) are discounted from analysis. Through calculating the logSD of the radius (R) of domains, heterogeneity of capillary spacing can be measured (Log_RSD). This is because of the lognormal distribution of R and the fact that in this case R is a measure of maximal diffusion distance from each capillary (Degens et al., 2002; Hoofd and Degens, 2013; Hoofd et al., 1985).

Another important parameter calculated was the number of domains around a fibre (DAF) that overlap with the fibre. This gave a better indication of oxygenation as it allows for the fact that diffusion can occur to fibres without direct capillary to fibre contact being established as is common in glycolytic muscle areas. The average of the DAF for a given image gives a value for local capillary to fibre ratio (LCFR) and can be calculated separately for each fibre type as the software allowed the different fibre types to be considered

separately. This value can be divided by the FCSA to provide a capillary fibre density (CFD) and as a result of the way it has been calculated, this value will be more sensitive to detect changes in capillary supply than would have been possible looking simply at parameters calculated without the consideration of domains (Degens et al., 2002).

4.4. Statistics

Analysis of data was carried out using SPSS (Statistics version 21, IBM, United States). Differences observed between variables collected at two time points (pre study and post study) ten years apart were compared using paired sample T-Test. Independent T-Tests were used where comparing variables that were not paired. Data collected on dependent variables were found to be parametric as was determined by using the Kolmogorov-Smirnov test. Stepwise regression analysis was also performed to test which factors acted as major determinants of indices of capillary supply. Unless stated otherwise, results are presented as mean \pm standard error about the mean (SEM). SEM was used to indicate the precision of estimated mean values generalized to the population rather than standard deviation (SD) which is a measure of variability around mean of a sample population. Results were compared from the pre-study and post-study groups to provide insight into changes that occurred over the ten year study as a whole.

5 – Results

5.1 – MyHC Composition Gels

Unfortunately results were not obtained for all of the subjects that were analysed through the method of capillary domain analysis. Many alterations were made to the protocol as early experiments produced spurious results to the point where eventually separation of the different MyHC isoforms was unachievable. Through altering all aspects of the protocol the conclusion was drawn that the samples provided had been improperly frozen causing denaturation of the protein or had denatured during freezing and thawing processes. This meant that confirmation of relative whole muscle biopsy MyHC isoform levels could not be carried out and that results and conclusions would have to be drawn from the cross-sectional histological morphology analysis alone.

5.2 – Cross-Sectional Morphology and Capillarisation Studies

5.2.1 – Inter-Rater Reliability

Due to the highly time consuming nature of the work and availability of resources, data were collected by more than one individual and combined to produce a larger quantity of data than would have been possible working alone. As the process of obtaining data relied upon tracing skill to convert micrographs to digitized traces, five identical micrographs were traced by all individuals involved in the data capture process to confirm no significant differences were apparent between separate traces of the same micrograph. For all of the data provided from the traces scores were deemed to be highly similar therefore data could be combined ($R^2 = 0.974$). The coefficient of variation (CV) was

determined for key parameters (FCSA and CD) also to provide further indication as to the reliability of combined work traced by separate individuals. The CV for FCSA was $\pm 3.7\%$ and for CD was $\pm 4.6\%$.

5.2.2 – Subjects

Subjects who provided samples for the study did so ten years apart and when providing samples both height and mass data was recorded. These were $178\text{cm} \pm 7\text{cm}$ and $80\text{Kg} \pm 8.2\text{Kg}$ respectively (presented as mean \pm standard deviation). There were no significant changes in the heights or masses of the individuals over the ten year period.

Per subject, at either one of the two time points (pre-study or post-study), 204 ± 96 fibres were analysed from the human *vastus lateralis* muscle. Data from the hybrid fibres was not included in the analysis as they accounted for less than 2% of the total fibres present and were only present in 3 of the 22 subjects. Results were compared from the pre-study and post-study groups to provide insight into changes that occurred over the ten year study as a whole.

5.2.3 – Fibre Morphology

Fibre number percentages (FNP) showed no significant change over the course of the ten year study. The percentage area that these fibres covered of the muscle cross section (Fibre Area Percentage (FAP)) also showed no significant change over the course of ten years.

Both the fibre number percentages and fibre area percentages were higher of type II to type I fibres although statistically significant differences between type I and type II fibres were only observed in the post study samples for both FNP and FAP ($P < 0.018$). The data are presented in Table 5.1.

FNP (%)	Type I Fibres		Type II Fibres		Significance Values between fibre types in post-study samples
	Pre-Study	Post-Study	Pre-Study	Post-Study	
FNP (%)	46.02 ± 2.84	43.76 ± 2.32	53.56 ± 2.81	55.56 ± 2.29	P=0.018
FAP (%)	45.50 ± 2.54	44.60 ± 1.98	54.34 ± 2.54	55.12 ± 1.97	P=0.016

Table 5.1 – Percentage values for the average fibre number percentages (FNP) and average fibre area percentages (FAP) for different fibre types for both pre and post study samples (10 years apart).

Fibre cross-section areas (FCSA) were not found to be significantly different between type I and type II fibres either pre or post study in any case. Slight declines were observed in both type I and type II fibres FCSA individually as well as all fibres FCSA collectively (type I and type II combined) between the pre and post study. This change was only significant however in type I fibres where the average FCSA of subjects declined by $276\mu\text{m}^2$ from $5056.8\mu\text{m}^2 \pm 169$ to $4780.0\mu\text{m}^2 \pm 167$ ($P < 0.05$). Between the pre-study and post-study group CV was calculated and found to be less than 5% for all fibres combined and type II fibres alone however it was found to be 9.8% for type I fibres alone.

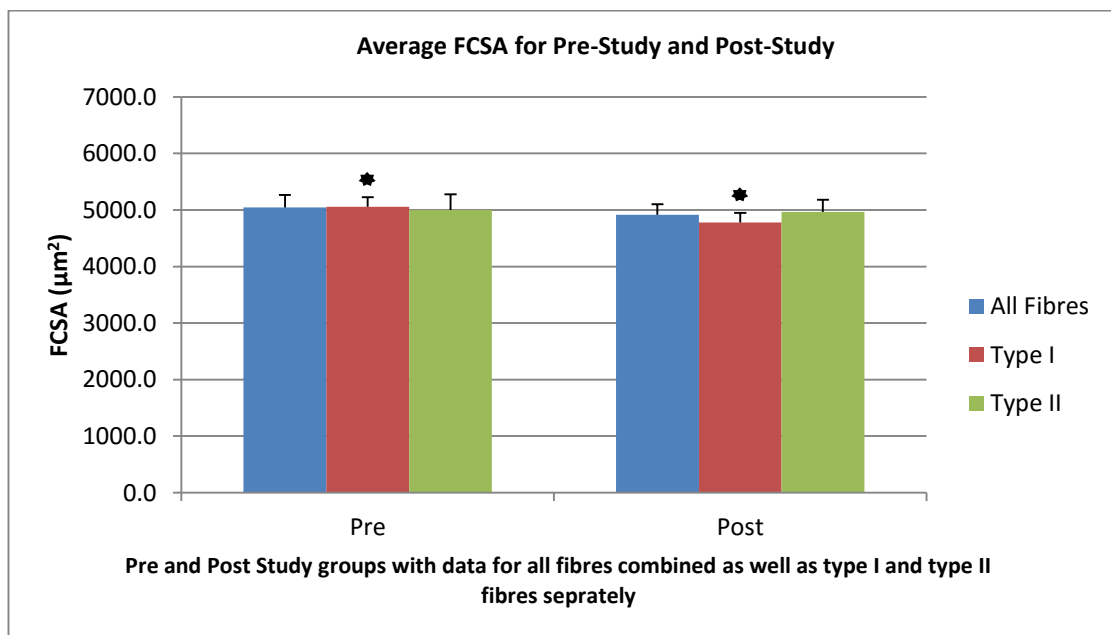


Figure 5.1 – Changes in average FCSA for all fibres combined as well as type I and type II fibres individually over the ten year period from pre-study to post-study time points. * indicates statistically significant values between type I fibres ($P < 0.05$).

The average standard deviation of the FCSA was compared between pre-group and post-group samples. This was done for all fibres collectively and for individual fibre types to see if there was any change in variation of the FCSA sizes. No significant changes were detected. These data are displayed in table 5.2.

Standard Deviation of FCSA (μm^2)	Standard Deviation of FCSA all fibres (μm^2)	Standard Deviation of type I fibres FCSA (μm^2)	Standard Deviation of type II fibres FCSA (μm^2)
Pre-Study Group	1374.82 \pm 127.24	1274.04 \pm 132.52	1298.59 \pm 134.72
Post-Study Group	1389.86 \pm 112.08	1280.57 \pm 122.76	1342.70 \pm 109.73

Table 5.2 – Means and standard deviations of FCSA presented for all fibres collectively as well as type I and type II fibres individually for both pre-study and post-study samples. Values presented and $\mu\text{m}^2 \pm \text{SEM}$

No significant changes were observed in the average measured fibre perimeters either of all fibres collectively or from individual fibre types over the duration of the study. Similarly no significant difference was observed between fibre types at either the pre-study time point or the post study time point. Data from the different age-groups was also found to not be significantly different between the age-groups with only a slight decrease seen. This was also true for findings between pre-study and post-study samples within the different age-groups. Findings for average perimeter of fibre types are displayed only as a collective and not for the different age groups due to similarity of results in each instance. In addition to this no significant changes were observed, although slight increases were seen with advancing age, in the average percentages of connective tissue (CT) deemed to be present from the cross sectional analysis. This can be found in table 5.3.

	Perimeter All (Type I & Type II) (μm)	Perimeter Type I (μm)	Perimeter Type II (μm)	Connective Tissue (%)
Pre-Study	287.3 \pm 4.8	286.0 \pm 3.8	286.5 \pm 6.4	7.65 \pm 0.6
Post-Study	283.2 \pm 6.2	279.4 \pm 5.7	285.3 \pm 7.1	8.39 \pm 0.6

Table 5.3 – Changes in average perimeter for all fibres combined as well as type I and type II fibres individually over the ten year period from pre-study to post-study time points. Connective tissue percentages from both pre and post study are also included. Values presented in $\mu\text{m} \pm \text{SEM}$

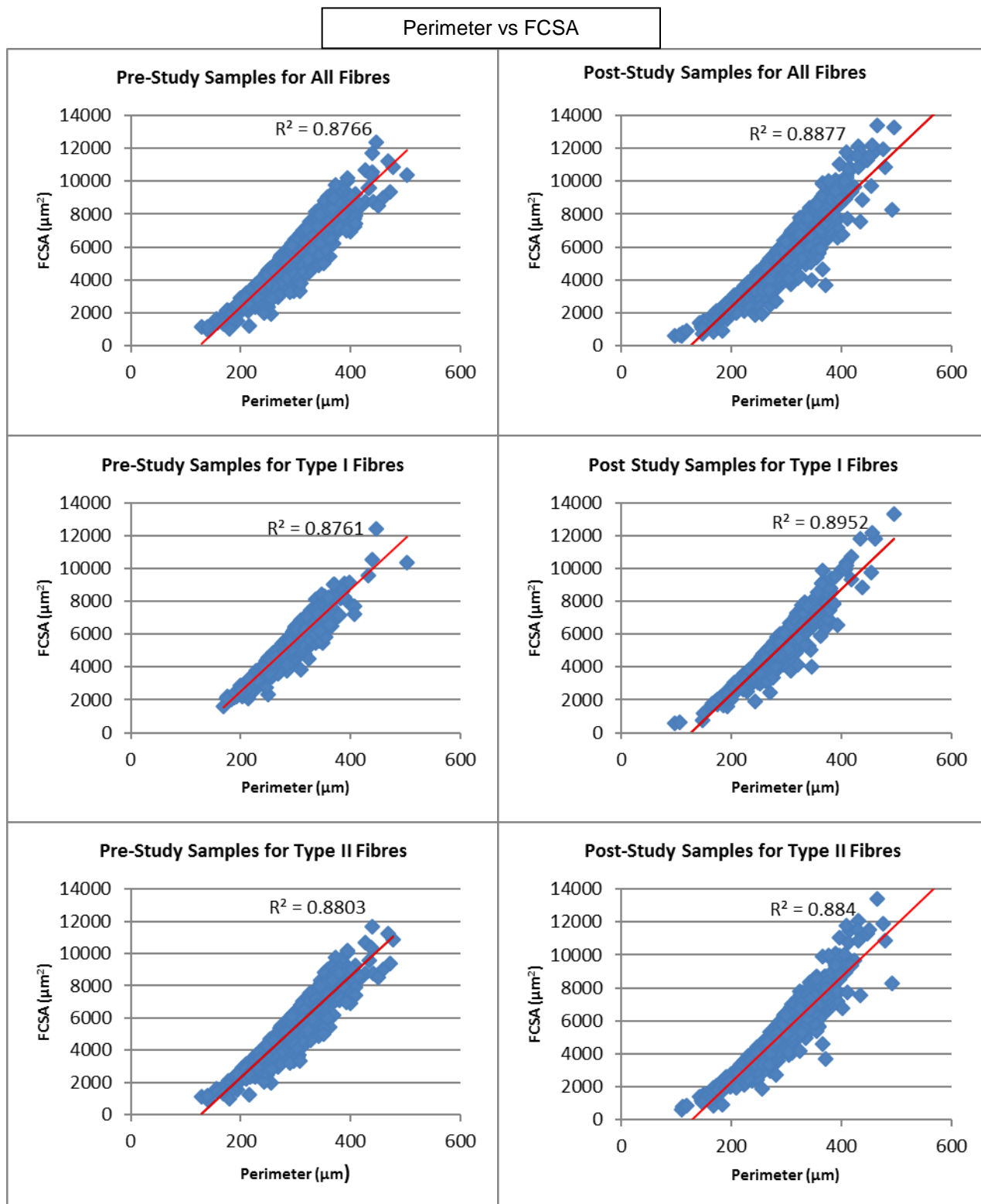


Figure 5.2 – Correlations between FCSA and perimeter for all fibres collectively and individual fibre types (type I and type II) for pre-study and post-study samples.

The perimeters of individual fibres were plotted against their corresponding FCSA to see the relationship between the two factors. Strong positive correlations very similar to one another were found in all instances when this was carried out for the different fibre types separately and collectively in the pre and post study group. This indicates that no significant differences or alterations in the angularity/circularity of the fibres of either type with advancing age or between fibre types. Figure 5.2 displays these results. Roundness index was also calculated and again no significant difference was found between pre-study and post-study samples for all fibres collectively or separate fibre types. Figure 5.3 displays results for roundness index.

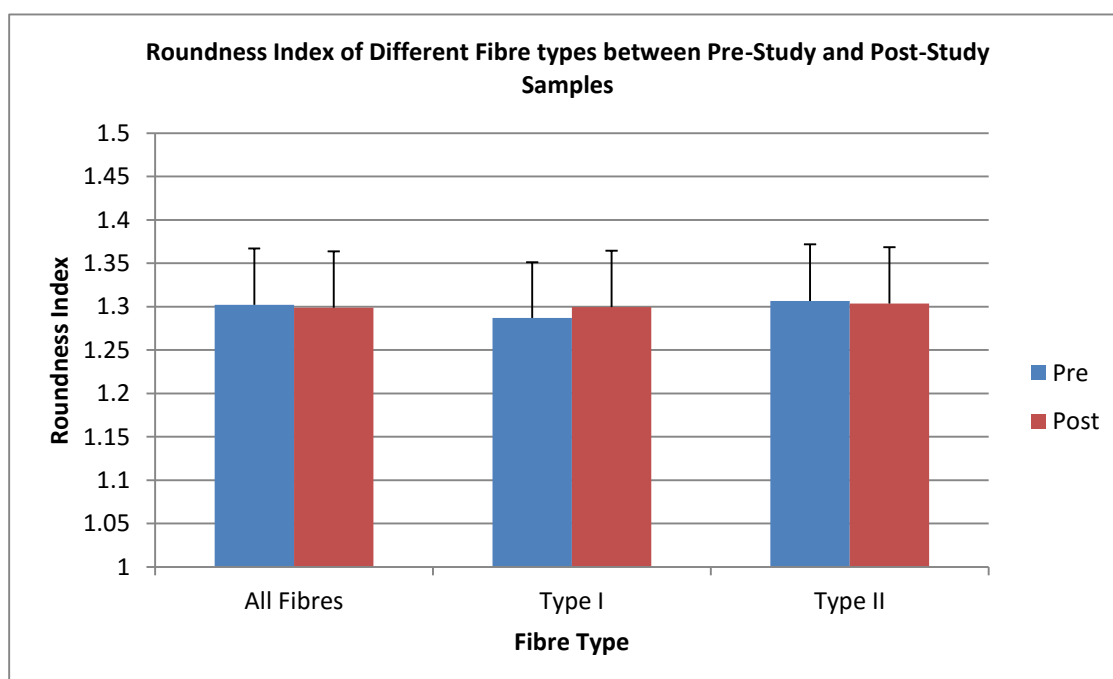


Figure 5.3 – Calculated roundness index for all fibres collectively as well as for individual fibre types in both pre-study and post-study sample groups

5.2.4 – Capillarisation

Indices of capillary supply that are irrespective of fibre type showed no significant differences between pre-study and post-study. Slight decreases were observed in both capillary density (CD) and the capillary to fibre ratio (C:F).

No decrease was observed in the heterogeneity of capillary spacing (Log_RSD). A slight, but insignificant, increase was observed in the capillary supply area indicative of the maximal diffusion distance not being significantly altered over the ten year period. CV was calculated for CD between the pre and post study groups and was found to be 7.8%.

Indices of capillarisation	Pre-Study	Post-Study	% Change	P-Value
CD (caps/mm ²)	247.2 ± 9.2	233.2 ± 9.55	- 5.7	0.220
C:F	1.47 ± 0.08	1.39 ± 0.07	- 5.6	0.198
Log _R SD	0.183 ± 0.004	0.184 ± 0.002	+ 0.5	0.813
Capillary Supply Area (µm ²)	3931.9 ± 137.3	4112.6 ± 180.9	+ 4.6	0.390

Table 5.4 – Changes in indicators of capillarisation between pre and post study including corresponding P-Values from repeated-measures t-tests.

No significant changes were observed for the number of domains around fibres (DAF) with slight declines being observed for all fibres collectively and the greatest decline being seen for type I fibres. Data for DAF are presented in table 5.5.

Fibre Type	DAF Pre-Study	DAF Post Study	% Change	P-Value
All	4.93 ± 0.16	4.74 ± 0.11	- 3.8	0.179
Type I	4.97 ± 0.16	4.67 ± 0.12	- 6.1	0.077
Type II	4.86 ± 0.17	4.76 ± 0.12	- 2.0	0.441

Table 5.5 – Changes observed in average the number of domains around fibres (DAF). The actual results are presented along with the percentage change and the P-Values obtained.

Local capillary to fibre ratio (LCFR) was one area where significant change was observed between the pre-study and post-study samples. An overall decline from 1.315 ± 0.072 to 1.195 ± 0.047 was observed when combining both type I and type II fibres. This decline was only small but significance between the two groups was found ($P < 0.05$). When considering the fibre types individually significance was only present in the decline observed for type I fibres ($P < 0.035$) with a decline from 1.325 ± 0.068 to 1.156 ± 0.052 . The observed decline in LCFR for type II fibres was not found to be significant (1.297 ± 0.079 to 1.208 ± 0.051).

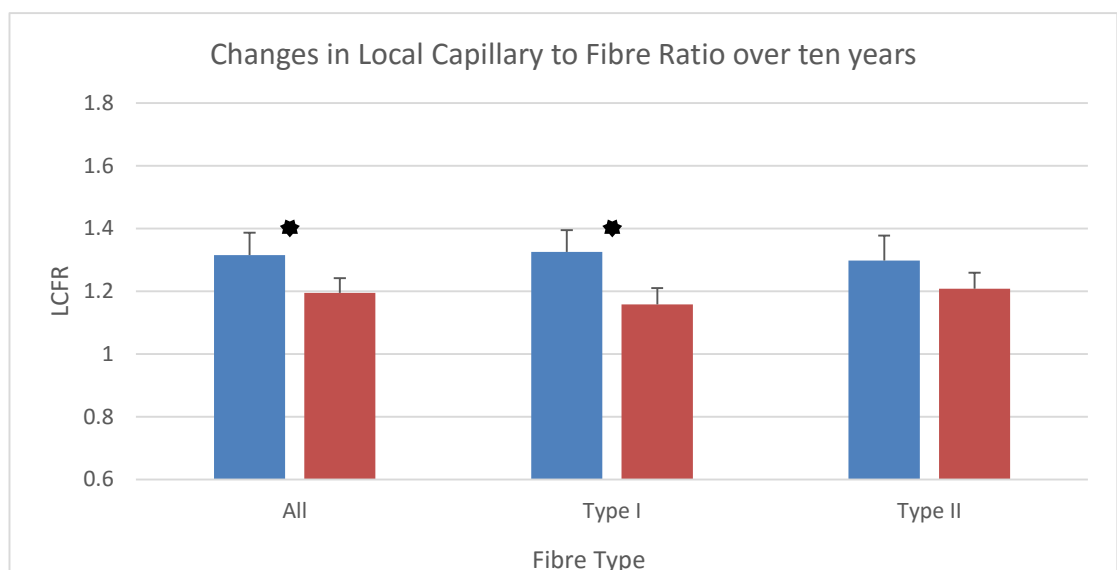


Figure 5.3 – Changes in average LCFR for all fibres combined and different fibre types individually from the pre and post study samples separated by ten years. * indicate statistical significance ($P < 0.05$)

Capillary fibre density (CFD), which is calculated from using the LCFR and dividing it by the FCSA of the fibre types in question (either individually or collectively) showed no significant changes even though declines were observed in both type I and type II fibres separately as well as collectively type I and type

II fibres combined. The largest decreases on average were again observed in type I over type II fibres but importantly none of the changes were significant.

Fibre Type	CFD Pre-Study	CFD Post-Study	% Change	P-Value
All	262.7 ± 9.8	244.6 ± 9.0	- 6.9	0.088
Type I	262.5 ± 10.9	240.0 ± 9.7	- 8.6	0.082
Type II	261.8 ± 9.4	247.5 ± 8.6	- 5.4	0.145

Table 5.6 – Changes observed in average Capillary Fibre Density (CFD). Values are in μm^{-2} . The actual results are presented along with the percentage change and the P-Values obtained.

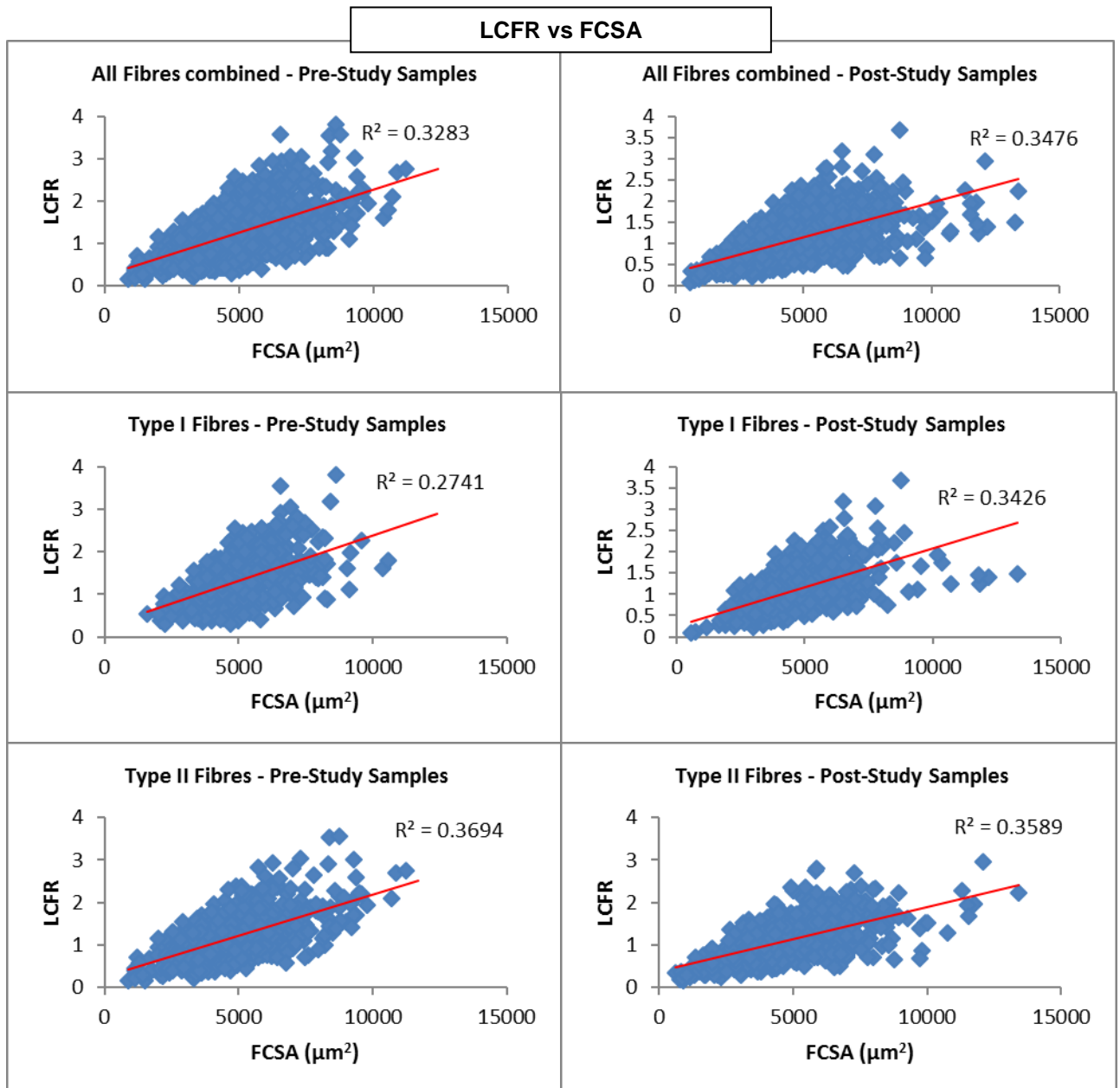


Figure 5.4 – Local capillary to fibre ratio (LCFR) plotted against Fibre Cross Sectional Area (FCSA) (μm^2) for all fibres collectively and for individual fibre types for pre-study samples and post-study samples. R^2 values indicate the strength of correlation between the two variables

The relationship between continuous indices of capillarisation and FCSA were analysed. Firstly the relationship of LCFR and FCSA was investigated by plotting the values against each other for individual fibres. A moderate positive correlation was present in all instances with little change present between the

strength of correlation between pre-study samples and post-study samples. The largest difference was observed between pre-study samples and post-study samples for type I fibres where the correlation between the two factors increased over the course of the ten year period. This finding was found to be statistically significant ($P=0.0468$) when these values were z-transformed and a student's t-test applied. Another relationship investigated was that of capillary fibre density (CFD) to FCSA. Unlike when LCFR was compared to FCSA and moderate positive correlations were present between the two variables, no correlations were present and no significant differences were present between the correlations between pre-study and post-study samples for all fibres collectively or separately.

The slopes of the regressions between FCSA and either perimeter, LCFR or CFD are summarised in table 5.7.

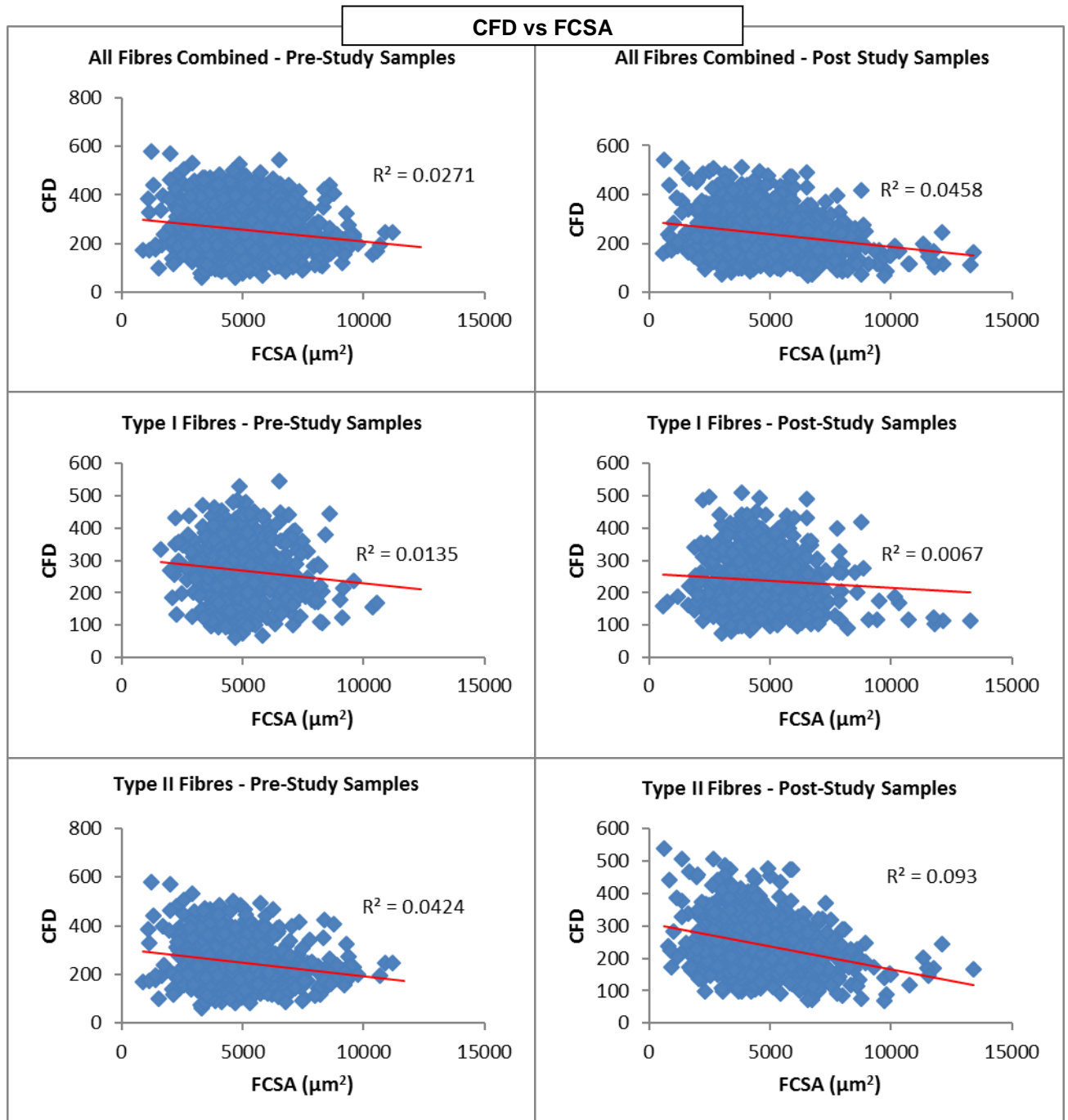


Figure 5.5 – Capillary Fibre Density (CFD) plotted against Fibre Cross Sectional Area (FCSA) (μm^2) for all fibres collectively and for individual fibre types for pre-study samples and post-study samples. R^2 values indicate the strength of correlation between the two variables.

Finally multiple linear stepwise regressions were performed between LCFR and shared factors fibre type and FCSA to ascertain which factor played a greater role in determining LCFR. Fibre type was found not to contribute but FCSA was found to be a key determinant for both the pre-study samples and post-study samples (shared contribution (SC) = 57.4% and 59% respectively). The same

process was carried out for CFD with neither FCSA nor fibre type found to be determinants in either pre or post study group.

Regression	Time-Point	All Fibres Combined	Type I fibres only	Type II fibres only
(Figure 5.2) FCSA vs Perimeter	Pre-Study	$y = 31.4x - 3920$	$y = 31.1x - 3740$	$y = 31.6x - 4050$
	Post-Study	$y = 31.8x - 4010$	$y = 31.9x - 4000$	$y = 31.7x - 4026$
(Figure 5.4) FCSA vs LCFR	Pre-Study	$y = 2 \times 10^{-4} + 0.247$	$y = 2 \times 10^{-4} + 0.255$	$y = 2 \times 10^{-4} + 0.240$
	Post-Study	$y = 2 \times 10^{-4} + 0.322$	$y = 2 \times 10^{-4} + 0.241$	$y = 2 \times 10^{-4} + 0.370$
(Figure 5.5) FCSA vs CFD	Pre-Study	$y = -1 \times 10^{-3} + 306$	$y = -8 \times 10^{-3} + 307$	$y = -1.1 \times 10^{-2} + 306$
	Post-Study	$y = -1 \times 10^{-2} + 289$	$y = -4.2 \times 10^{-3} + 258$	$y = -1.4 \times 10^{-2} + 307$

Table 5.7 – Equations for slopes of regressions seen in pre-study and post-study for all fibres combined as well as type I and type II separately. This has been shown for FCSA vs Perimeter (Figure 5.2), LCFR (Figure 5.4) and CFD (Figure 5.5)

6 – Discussion

6.1 – Cross-Sectional Studies

6.1.1 – Subjects

Training levels over the 10 year period of the individuals analysed were maintained to levels higher than those expected of age-matched sedentary individuals. As no significant changes were observed in the height and weight data collected over the 10 year period it can be assumed that the subjects did, as declared, carry out their training programmes as declared and maintained health and fitness levels making them appropriate for the study.

The subjects involved in the study included some (5) subjects who were aged below 50 at the start of the study (pre-study) however this study group was still considered to represent an aging cohort as the average age of subjects involved in the study was still higher than 50 and all were older than 50 when the final biopsies were taken (post-study). Furthermore data were being analysed over the course of a ten year aging period allowing differences to be seen before and after the ten year study.

The cohort of master athletes involved in this study may be considered to be atypical to that of the population as it can be argued that the higher than average health and physical performance levels may be a product of self-selection, lifestyle differences and a genetic make-up that lends itself to maintaining muscular function better into older age. However the improved health and survival rates observed in master athletes compared to sedentary individuals mirror those seen when comparing untrained to life-long trained rats with similar a genetic constitution (Edington et al., 1972). Furthermore VO_{2max}

of master athletes has been shown to revert back to levels similar to those seen in age-matched non-athletes along with increases observed in normally sedentary individuals who undergo endurance training (Pollock et al., 1997). This would suggest that the decline in muscle function is largely attributable to disuse and is reversible with appropriate training (Booth et al., 2011) meaning that findings from the study on this cohort can be applied to the general population and should not apply only to this cohort.

6.1.2 – Fibre Morphology

No significant changes were observed over the ten year period in either FNP or FAP (Table 5.1) suggesting that no fibre type transition occurred toward one or other fibre type as a result of ageing over the ten year period. In normal sedentary individuals, as ageing occurs, it is expected that a decline in both the FNP and FAP of type II fibres will occur with a corresponding increase being observed in type I fibres (Evans and Lexell, 1995). In both the pre and post study the FNP and FAP of type II fibres was higher than type I fibres although this difference was only significant ($P < 0.018$) in the post-study group. The fibre type composition being in favour of type II fibres to type I fibres of around 54% to 44% is similar to that seen in previous studies of young men (Johnson et al., 1973; Kirkeby and Garbarsch, 2000). The similarity seen to this, as well as the lack of a statistically significant change in fibre type composition between pre-study and post-study samples, suggests that no specific fibre type transition is present. As the subjects being analysed are power athletes and train in a manner that requires use and regular activation of type II motor units this result implies that motor units in these individuals are not being lost as would

normally be observed in sedentary individuals with ageing and that the motoneuron degeneration expected to happen is being attenuated by the impact of resistance and power based training. Further work would be needed to confirm that the relative MyHC isoform levels are indeed not altered which could be confirmed by methods such as the MyHC composition gel electrophoresis that was intended to have been carried out.

As seen in figure 5.1 slight declines were observed in FCSA however this decrease was only significant when considering type I fibres alone ($P < 0.05$). This finding had an effect size of 0.22 from the pre-study group to that of the post-study group. When compared with the effect sizes seen for all fibres combined and type II alone (effect size < 0.05) it is clear that the type I fibres were impacted the most. This result was unexpected as ageing is normally associated with an overall decline in FCSA of all fibres with particular decreases seen in type II fibres. Type I fibres would be expected to be largely unaffected over a ten year period and even in some instances increase due to compensatory hypertrophy occurring as type II fibres atrophy (Lexell et al., 1988). As was observed in FNP and FAP the usual decline in type II fibres and transition to type I fibres seen in sedentary individuals during ages does not present itself with the group of power-trained master athletes being analysed here. Possible explanations for this include the fact that the compensatory hypertrophy, that is a resultant effect during ageing as selective type II fibre atrophy occurs, may not be required as type II fibres appear to be better maintained in the master athlete group in question. This again could be as a product of the resistance and strength based training regimes that were undertaken. Another area where

the subjects in question showed difference previous studies into ageing muscle was with the lack of a significant increase in variation of fibre sizes represented by standard deviation (Table 5.2). As part of the compensatory hypertrophic response of type I fibres to the selective type II fibre atrophy, variation is expected to increase especially in type I fibres but also as a whole as type II FCSA decreases and type I FCSA increases but no such effect was observed within this group of individuals. Furthermore as denervation occurs muscle fibres tend to become less circular as they atrophy but, as was represented by the relationship between FCSA and fibre perimeter along with the roundness index scores (Kirkeby and Garbarsch, 2000), no significant alterations to the general angularity of fibres was detected (Figure 5.2 and 5.3). This may be an important factor in maintaining oxidative capacity in muscle as the transport of oxygen from capillaries to the mitochondria can be impaired because of a loss of the optimal circular shape of muscle fibres (Mathieu-Costello and Hepple, 2002). In addition it is normal with ageing to see an increase in the percentage of connective tissue present increase as fibrosis levels increase and fibres atrophy (Degens, 2007). A slight but insignificant change was observed in this study again supporting the hypothesis that exercise can act to preserve the state of the muscle tissue (Table 5.3).

Overall, the general fibre morphology did not appear to deteriorate or change as expected to do so with age although more work is needed to be carried out to further confirm this. Studying the effects over a longer time period and with a larger cohort would allow for even more in-depth analysis to be carried out

and look at the progressive nature of ageing seeing whether the effects of training can attenuate the sarcopenic phenotype.

6.1.3 – Capillarisation

Similar to the findings in the fibre morphology there was generally a lack of significant difference observed between the pre-study samples and post-study samples in many of the indices of capillarisation. CD and C:F ratios did show a slight decline, associated with the corresponding slight increase in maximal diffusion distance (represented by Complete Domain Area), however these change were not statistically significant (Table 5.4). Calculated effect sizes supported this conclusion for CD where it was found to be less than 0.05. This was not in line with some studies (Proctor et al., 1995) that indicate a reduction in the number of capillary contacts present during fibre atrophy of which the rate is increased with ageing. There was no decrease in the heterogeneity of capillary spacing (Log_{RSD}) (Table 5.4) suggesting that while overall capillary numbers may have decreased, this process is not random. It is in fact likely that this is an adaptation of muscle tissue to cope with some of the age-related changes in capillary supply to maintain adequate whole muscle tissue oxygenation levels and functionality as this is an indicator of muscle oxygenation (Degens and Veerkamp, 1994; Degens et al., 1994). As the decline is slight and not statistically significant it is possible that the normal rate of capillary decline is being prevented by the higher than normal physical activity levels.

CD and C:F are parameters that do not take into account oxygen supply from capillaries not in direct contact with fibres and so it is important to consider the

parameters that do so. Once again no significant change – despite a slight decrease – in DAF was observed (Table 5.5) over the ten year period again suggesting maintenance of oxidative supply. As DAF is a discontinuous variable an even more sensitive index (LCFR) which has the benefit of being continuous was considered and showed significant changes when all fibres were analysed irrespective of fibre type over the ten year period. This change was more pronounced in type I fibres and no statistical change was observed in type II fibres (Figure 5.3). This result may however, be the product of the statistically significant decrease seen in type I FCSA (Figure 5.1) over the study as it was demonstrated that LCFR and FCSA show a positive correlation (Figure 5.4). This conclusion is supported by the fact that no statistically significant decrease in CFD was apparent (Table 5.6) as CFD is calculated by dividing the LCFR by the FCSA. CFD is perhaps the most sensitive parameter available as it takes into account indirect capillary supply as well as fibre size along with the overall density of capillaries without being heavily determined by the FCSA itself as was found in the stepwise regression analysis.

The lack of a statistically significant decrease in CFD and maintenance of Log_{RSD} supports the hypothesis that the capillary network is better maintained in individuals exhibiting good levels of physical activity. Furthermore the fact that only statistically significant declines were observed in type I fibres indicates that the type II fibres predominantly for power based activity are being very well maintained in their capillary supply and therefore oxygenation levels. This can be assumed even without the availability of actual oxidative capacity data as capillarisation has been shown to be significantly related to this (Mathieu-

Costello and Hepple, 2002; Bekedam et al., 2003) although having this data in addition would be beneficial.

6.2 – Future Work

To support the findings made in this study it would be ideal to carry out analysis on a larger cohort of subjects with a greater variety of genetic backgrounds (all considered here were Finnish master athletes) to see whether the findings are mirrored elsewhere. It would also be of great interest to see whether the same effects would be observed in master athletes whose main form of training and competition is endurance-based to see whether there is less of the apparent protective effect seen of the type II fibres in these power-based master athletes that is not seen in sedentary individuals. Differences in gender would also be another area of interest. By analysing a larger cohort it would be possible to sub-divide the group of master athletes by age groups further so that changes (or lack thereof) over a given time period could be examined in greater detail to examine the progressive nature of sarcopenia and ageing.

Data on oxidative capacity through succinate dehydrogenase (SDH) staining would also be a great addition to the current data provided as well as being able to confirm the relative MyHC isoform levels at in the entire muscle biopsy through gel electrophoresis to see whether the cross-sectional analysis approach provides sufficient data to determine fibre morphology and capillarisation at the whole muscle level. Studies into the number and functionality of satellite cells within master athletes muscle tissue compared to sedentary issues would be yet another area that would provide insight into the effects exercise has upon retaining normal muscle function during ageing.

7 - Conclusion

The aim of the study was to understand the contribution age-related decline in physical activity levels has upon the skeletal muscle phenotype and to the process of sarcopenia. In order to achieve this aim the confounding variable of the normal age-related decline in physical activity levels associated was eliminated by studying a group of individuals (master athletes) who retain much higher than average physical activity levels into old age.

The ageing process was studied by gathering muscle biopsy samples from these individuals at two time points, ten years apart from one another to see how various indicators of muscle quality changed over this time period. It was hypothesised that due to the physical activity levels of these individuals being far greater than those seen in age-matched sedentary individuals; the normal changes seen in muscle associated with ageing would be less severe and in some instances not present.

As discussed in both the results and discussion sections various indicators of muscle tissue quality were assessed and, in general, no significant differences were seen between the two separate time points, ten years apart, indicating that exercise does play a vital role in attenuating the normal age-related muscle wasting processes portrayed through both fibre morphology and capillarisation.

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